

RESPIRATORY STUDIES OF SINGLE CELLS. I. METHODS¹

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For a detailed study of respiratory events in single cells, even large ones such as the commonly used marine eggs or protozoans, a stability of a few $\mu\mu\text{l}/\text{hour}^2$ or better and a sensitivity down to a fraction of a $\mu\mu\text{l}$ are desirable. Only one method (Zeuthen, 1943) has been described so far that approaches this order of magnitude. By modifying and reducing drastically the conventional Cartesian diver respirometer (Linderstrøm-Lang, 1937, 1942, 1943; Linderstrøm-Lang and Glick, 1938; Boell, Needham and Rogers, 1939; Holter, 1943), he was able to achieve a stability of 0–20 $\mu\mu\text{l}/\text{hour}$ and a sensitivity of about 20 $\mu\mu\text{l}$. With this 0.1 μl diver he measured the oxygen consumption of a single polychaete worm egg and of single amoebae. In another series the method was used in a study of the single, half, whole and syncytial egg of an ascidian (Andresen, Holter and Zeuthen, 1944; Holter and Zeuthen, 1944).

Large single amphibian eggs have been studied by means of larger and less sensitive modifications of the conventional Cartesian diver (Zeuthen, 1946).

In this paper we present a method for micro respiratory studies of single cells that so far has yielded results with a sensitivity of about 0.2 $\mu\mu\text{l}$ and a stability (drift) from 0–10 $\mu\mu\text{l}$ per hour. The method is in some respects simpler than the 0.1 μl Cartesian diver and seems to offer possibilities for further refinements. It is to be hoped it will prove its usefulness among flotation methods for measuring respiration, the development of which has been so successfully carried out at the Carlsberg Laboratory.

I. THE RESPIROMETER

In 1911 Krogh developed a method for micro gas analysis which he named microscopical gas analysis and which consisted essentially of measuring the diameter of gas bubbles under the microscope before and after absorption of the CO_2 and O_2 . In 1941, it seemed to one of the authors that it might be possible to place a cell in a small chamber full of water together with a bubble of oxygen. The chamber would be in pressure equilibration with the outside water bath by means of a fine capillary connecting space. The cell would consume the bubble and the rate of shrinkage could be measured through the microscope. The stability of a bubble in such a system without a cell was tested, using a chamber identical with the ones used at present, and was found very good. However, as the bubble shrinks from the oxygen consumption the surface tension builds up an increasing gas pressure in it, according to the relation $P = \frac{4T}{D}$, where T = the

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² One $\mu\mu\text{l}$ is defined as one millionth of one cubic millimeter = liter $\times 10^{-12}$.

surface tension and D the diameter. If T is taken as 73 dynes/cm., the pressure in the bubble due to the surface tension will be $\frac{2200}{D}$ mm. Hg when D is measured in microns. Hence, a 10 μ bubble has an overpressure of 220 mm. Hg, a 100 μ bubble an overpressure of 22 mm. Hg, etc. It is therefore clear that this principle can only be applied on a manometric basis where the pressure is so adjusted that the bubble volume is kept constant. As in any manometric method, the change in pressure will reflect the oxygen consumption when the volume of the gas and liquid content of the chamber are known. The problem therefore became how to know most accurately that the bubble was being maintained at constant volume. By microscopic measurement of the bubble the volume could be estimated with an accuracy of no more than three per cent, which was not very satisfactory. A much more sensitive way of ascertaining the volume constancy of our bubble would be to make use of the extreme accuracy whereby the density of minute objects can be determined by flotation (Linderstrøm-Lang and Lanz, 1938). The problem would then be to stick the bubble in the respiration chamber onto a hydrophobic minute weight and then to regulate the pressure so that the bubble would balance the weight suspended in the water.

In the Cartesian diver the constancy of the gas volume of the chamber is ascertained by floating the whole chamber. In the present system a minute reference diver is floated in the respiratory chamber containing the cell. The gas phase is less than one per cent of the water phase, and the pressure effect by respiratory CO_2 can, at most, amount to about three per cent of the changes in the oxygen tension, even in acid media. This is due to the much higher solubility of CO_2 than oxygen. At slight alkalinity the CO_2 pressure will remain practically zero. Usually, therefore, no special provision for CO_2 absorption need be made.

A. Principle of the method

A minute respiration chamber is filled with a medium which communicates to the same medium on the outside through a fine capillary space. This space serves as a diffusion barrier, but permits free pressure transmission to the chamber from the outside medium, upon which controlled pressure can be applied. The respiration chamber contains, besides the cell, a little bubble adhering to a minute hydrophobic weight. The pressure is adjusted so that the bubble always just suspends the weight to a constant height in the respiratory chamber. We have then a constant volume system, where the pressure changes, read on the manometer, multiplied by a factor calculated from known constants give the oxygen consumption. The CO_2 is effectively held in solution by the large liquid to gas ratio of the system and amounts to three per cent or less of the factor.

B. Construction (Fig. 1)

The apparatus is simple and can be assembled in almost any laboratory from conventional equipment. It consists of the respiratory chamber assembly with the reference diver, accessories for making the reference diver and for charging the respirometer, the manometer with pressure control, the water bath, and optical equipment.

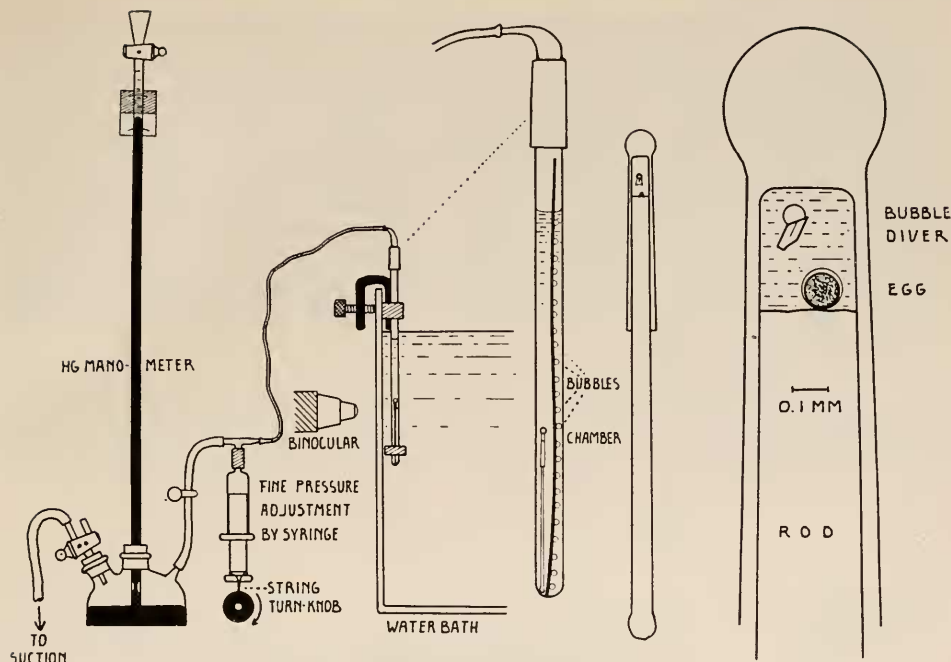


FIGURE 1. Respiration measurement of single cell by means of reference diver.

1. Respiratory chamber assembly

This consists of the chamber, the chamber tube, and the plastic reference diver.

a. Preparation of glass tubing. The respiration chamber is made from gently heated, soft glass.³ We have not been able to use cleaning solution or detergents in the finished chambers as this would often produce drastic toxic effects on *Paramecium*, even after lengthy boiling and rinsing in water. All tubing is therefore cleaned before making the chamber. The roundest possible (check by micrometer) soft glass tubing is selected (2.5–3 mm. OD), and is cut into 50 or so pieces 10 cm. long. They are then put in a beaker with 2% Dreft (or other detergent), boiled for a few minutes and left covered by the same solution for future use.

b. Making the respiration chamber (Fig. 2). A soft glass rod, 2–3 mm. thick and as round as possible, is heated evenly by rotation in a flame and pulled out straight to form a symmetrical cone with a long taper, reaching the desired diameter of about 0.3 mm. while still tapered. The desired diameter is determined by a micrometer and the rod cut off at that point, with the least possible chipping. We have found a triangular India honestone to be a better glass cutter for these dimensions than a sharp Carboloy steel edge (which cuts the finger better than the glass). The thick end is cut off and flamed to a knob.

³ It was found that in the dimensions here used, a freshly made respiration chamber of Pyrex glass is highly toxic to some cells, *Paramecium*, for instance.

A glass tube is removed from the detergent solution and rinsed first under the faucet and then with distilled water. The outside is dried and the inside water is shaken out. The tube is rotated and heated evenly and pulled out to a long taper. The wide end is cut off and the rod stuck into it as far as it will go. It is essential to check under the dissecting microscope to see *that colored Newton rings are visible at the end of the rod all around the circumference extending a millimeter or more back from the end*. This is the only criterion of tightness and hence of the efficiency of the diffusion barrier between the chamber and the outside. It is usually necessary to try several glass tubes before a satisfactory fit is obtained. It is useless to try rods or tubing unless they are pulled out straight and form a symmetrical cone at the thick end. Unless these conditions are fulfilled the taper will not be round.

The rod is stuck rather loosely into the tubing, which is melted off in a micro flame⁴ a few millimeters from the rod end (Fig. 2). In this way combustion products from the flame cannot enter the chamber. With the chamber still loosely stuck onto the rod, the sealed end is rotated and melted to form a sym-

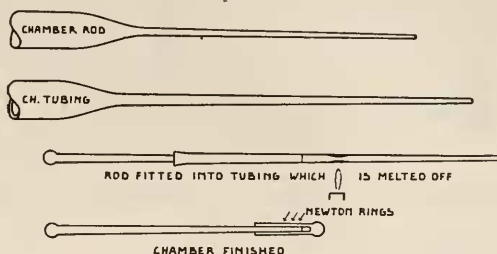


FIGURE 2. Making the respiration chamber.

metrical knob. The rod is pressed in carefully and the chamber is measured. The length should be 1–1.5 times the diameter. If it is too long loosen the rod and heat the knob again, until the desired length of chamber is obtained. The sleeve of the chamber is cut off at a total length of about 5–6 mm. The chamber is now ready for use.

c. The chamber tube (Fig. 1). The respiratory chamber rests during the experiment in a chamber tube about 0.5×12 cm., which is fastened to a clamp by means of a section of rubber tubing, so that it can be moved in all directions. The clamp screws onto the front of the water bath. A fine polyethylene tubing, 1.5–2 mm. OD, connects the tube with the pressure system. The tube also holds a tension equilibrating device, consisting of a piece of Saran filament, 1.5–2 mm. thick, pitted every 3 mm. with a sharp needle point. When the pressure is lowered to generate the bubble on the diver, bubbles will also form at each pit and this will facilitate the establishment of the same gas tension in the water outside and inside the respiration chamber.

d. The reference diver (Fig. 3). A piece of Saran tubing,⁵ 10 cm. long and about 5 mm. OD, is rotated and very gently heated 10 cm. or so above a low Bunsen flame. When carefully done the tubing will turn clear and melt together

⁴ S. S. White (Philadelphia, Pa.) Orthodontic Burner No. 6.

⁵ Manufactured by the Dow Chemical Co., Midland, Michigan.

and form a thinner capillary with a fine hole. One end is quickly clamped and the other end pulled out and stretched to a length of several meters, forming a fine filament. A piece 50–60 μ thick with a 1–2 μ hole is selected from the filament. It is wound on a card for safe keeping. While on the card the filaments are measured under a microscope and a piece 2–3 cm. long is cut out, having a uniform, accurately known diameter and a small central hole. The piece is fastened at one end by Scotch tape to a piece of a white plastic millimeter ruler attached to the top of a number 10 rubber stopper, which serves as a convenient "working table." A splinter of a new xylene-cleaned razor blade is twisted off (Fig. 3) and serves as a knife. Before cutting the diver under the binocular a little distilled water is run down on the filament and wiped off with clean gauze.

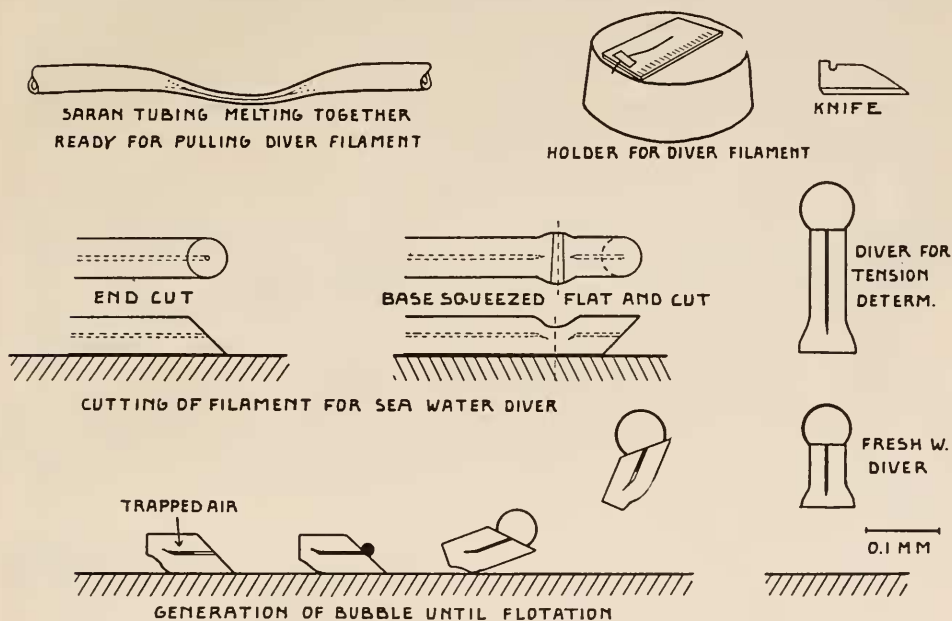


FIGURE 3. Preparing reference diver and bringing it to flotation.

We have used two types of reference divers. One has the top cut obliquely to prevent the bubble from hitting the chamber wall, which is hydrophobic in sea water. The other type has a straight cut top and can usually be used in fresh water, which wets the chamber walls.

To cut the oblique diver, the holder is placed under the binocular and the filament held down with a washed and dry finger. The end is cut with the very tip of the razor blade, slanting it at a 45° angle against the table. The diver filament must be seen to have air in the central hole where it is cut. The cut must be clean and sharp, leaving the hole open. Roll the filament over 180° and squeeze the base of the diver flat with the back side of the knife point, so that it is seen that the capillary closes. Then cut straight. The minute diver usually adheres to the knife point and is transferred directly into the already loaded respiration chamber. It can also be stored for some time in a drop of

water without losing the air that is trapped in the capillary. It can then be transferred somewhat more readily from the drop by means of a braking pipette (Holter, 1943; Claff, 1947) or micrometer burette provided with flexible plastic micro tubing ending in a fine glass tip.

A straight cut diver for fresh water or tension work is basically made the same way.

2. Manometer and pressure control (Fig. 1)

For the pressure measurements we have employed a closed mercury manometer of the same type as used in the Van Slyke-Neill manometric blood gas apparatus. With oxygen-charged water it gives a useful scale of about 600 millimeters. This type of manometer obviates the need for thermobarometer control and possesses a large pressure range, which seems desirable for this method.

The manometer is prepared as follows.⁶ Suction is applied to the top, and the mercury is brought all the way up by slanting the manometer. A little ethylene glycol is run down in the tube on top of the mercury. Most of it is removed again by tilting and applying suction from the top. With the top stopcock closed the barometer is evacuated by the suction pump⁷ to get rid of the gases on the walls of the barometer tubing and in the ethylene glycol. The ethylene glycol is allowed to collect at the top and is carefully removed by suction from the top of the tilted manometer. It is necessary to get rid of most of it or it will collect on the meniscus and interfere with the reading.

Check that the barometer reads the correct barometric pressure by measuring the distance between the top meniscus and the lower surface in the mercury container. It should check within one or two mm.

Accurate reading of the barometer is greatly facilitated by the following simple device (Fig. 1). A white card with black backing at the top is clipped onto the barometer tube. For a reading the card is slid so that the black backing runs slightly above the meniscus. The meniscus will then stand out sharp black against a white background.

The pressure has one coarse and one fine adjustment. The coarse adjustment is provided either by applying vacuum from a steadily running vacuum pump or admitting air pressure by turning the two-way stopcock.

Fine adjustment is provided by the 5 cc. syringe arrangement shown in Figure 1. The syringe is kept lubricated by applying heavy oil around the barrel seal. The plunger must move smoothly without sticking.

3. Water bath

An ordinary large (40 liter) glass aquarium tank is used as water bath. It is insulated by Celotex or several layers of corrugated paper, except for the upper six or seven centimeters front and back. In front of the bath rests a horizontal binocular, preferably with rack and pinion up and down, and in back of the bath opposite the microscope a small fluorescent lamp. The water bath must be kept within $\pm 0.01^\circ$ C. or better of the desired temperature. If an automatic thermostat is used, it is imperative to check the magnitude of the cycling on a Beckmann thermometer during an hour's time to be sure that it does not exceed

⁶ For more detailed description see Peters and Van Slyke, 1932, p. 273.

⁷ A laboratory suction-blower unit will do.

0.01 degree. The heating and cooling sources should be as gentle as possible. An infrared lamp suspended above the water bath and connected with a rheostat makes an excellent source of heat, and a mere trickle of cool water on the stirring propeller may serve as a cold source. By regulating the rheostat the temperature can easily be controlled within the desired limits, even by hand.

4. Optical equipment

For observing the cell and the reference diver, any type of binocular dissecting microscope mounted horizontally can be used. It must be provided with an ocular micrometer scale which can be calibrated against the grid of a blood counting chamber or even against a millimeter ruler stuck into the water bath. From 40 up to 100 times magnification has been found satisfactory.

For accurate measurement of the diver filament, egg sizes, etc., an ordinary microscope with an ocular micrometer is necessary. Another dissecting microscope is used for loading the chamber, cutting the reference diver, etc., and it is convenient to have it also provided with an ocular micrometer.

II. THEORY

The system is in principle a simple constant volume apparatus, where the constancy of the gas volume, as in a Cartesian diver, is determined by flotation rather than by reading a meniscus, but where the CO_2 is held in the medium rather than being separately absorbed.

A. Equations and constants

The oxygen consumption or CO_2 evolution is given by equations 1, 2 and 3, when only one gas is considered.

The following symbols will be used: V_B = volume of gas bubble; V_L = volume of liquid; P = observed pressure; W = water vapor tension; N = nitrogen tension; S = bubble pressure produced by surface tension;⁸ T = absolute temperature; ΔP = observed pressure change; α = solubility coefficient for the gas that changes; F = the chamber constant. STPD means "measured dry at standard temperature and pressure."

Gas volume (STPD) at start is

$$V_B \frac{273}{T} \left(\frac{P - (W + N - S)}{760} \right) + V_L \alpha \left(\frac{P - (W + N - S)}{760} \right) = A. \quad (1)$$

Gas volume (STPD) at end is

$$V_B \frac{273}{T} \left(\frac{P - (W + N - S) - \Delta P}{760} \right) + V_L \alpha \left(\frac{P - (W + N - S) - \Delta P}{760} \right) = B. \quad (2)$$

$$\text{Gas consumed (or produced)} = A - B = \Delta P \left(\frac{V_B \frac{273}{T} + V_L \alpha}{760} \right), \quad (3)$$

⁸ See page 157.

which is the well-known formula for calculating the flask constant in a Warburg manometric apparatus.

In a system where CO_2 is not separately absorbed, as in our system, an expression for the oxygen consumption ΔO_2 in terms of the pressure reading ΔP is given by the following equations:

$$\Delta V_{\text{O}_2} = \Delta P_{\text{O}_2} \left(\frac{V_B \frac{273}{T} + V_L \alpha}{760} \right), \quad (4)$$

$$\Delta V_{\text{CO}_2} = \Delta P_{\text{CO}_2} \left(\frac{V_B \frac{273}{T} + V_L \alpha}{760} \right). \quad (5)$$

Assuming $RQ = 1$,

$$\Delta V_{\text{O}_2} = \Delta V_{\text{CO}_2}, \quad (6)$$

$$\Delta P = \Delta P_{\text{O}_2} - \Delta P_{\text{CO}_2}. \quad (7)$$

Solved for ΔV_{O_2} the final equation gives the oxygen consumption:

$$\Delta V_{\text{O}_2} = \Delta P \left(\frac{1}{\frac{760}{V_B \frac{273}{T} + V_L \alpha_{\text{O}_2}} - \frac{760}{V_B \frac{273}{T} + V_L \alpha_{\text{CO}_2}}} \right) = \Delta P \times F. \quad (8)$$

It will be noted that pressures which remain constant, such as those contributed by water vapor, nitrogen and surface tension of the bubble, cancel out in equations 1 and 2 and are of no influence as long as they remain constant. (See derivation of equation 3 in Dixon, 1943.)

Besides the pressure difference ΔP read on the manometer, we must know T , V_B , V_L , α_{O_2} , and α_{CO_2} . T is equal to the water bath temperature. V_B is very small and can be estimated closely enough from its measurement in the ocular eyepiece. It can be better estimated by knowing the volume per millimeter of the diver filament and calculating the volume of the diver from its measured length. With a specific gravity of Saran of 1.7, the bubble volume will be the diver volume times 0.7.

V_L is obtained from measuring in the binocular the diameter and the height of the chamber, allowing for the rounded upper corners. In order to save repeated calculations a chart is prepared, where on a log log paper the diameter from 0.1–1 mm. is plotted against the volume per millimeter height, which at 0.2 mm. diameter is 314,000 μl and at 0.8 is 503,000 μl . A straight line is drawn through these points. The volume/mm. read on the graph multiplied by the height in millimeters gives the chamber volume in μl . The cell may conveniently and without significant error be considered water. The diver volume plus the bubble volume is usually smaller than the error in the estimation of the chamber volume and can then be ignored. Usually, therefore, V_L can be taken equal to the chamber volume.

The absorption coefficient of oxygen, α_{O_2} , for fresh water may be found in any handbook, and for sea water of different salinities and temperatures it may be found by interpolation of data given in Sverdrup, Johnson and Fleming (1942).

The absorption coefficient at different temperatures for carbon dioxide (α_{CO_2}) in acid media can be found in standard handbooks and for acid sea water in the above mentioned book. In neutral or alkaline media the CO_2 retention can without much difficulty be determined, as described below.

B. The pressure effect of CO_2 production

From equations 1 and 2 it can easily be shown that the ratio $\frac{\Delta P_{O_2}}{\Delta P_{CO_2}}$ depends upon the ratio of the solubility coefficients and the ratio of the liquid volume to the gas volume. In an all gas phase the carbon dioxide will of course exercise its full pressure, so that $\frac{\Delta P_{O_2}}{\Delta P_{CO_2}} = 1$, whereas in an all liquid phase the ratio becomes $\frac{\Delta P_{O_2}}{\Delta P_{CO_2}} = \frac{\alpha_{CO_2}}{\alpha_{O_2}}$. From Figure 4 it will be seen that when the gas volume gets smaller than 1% of the liquid volume, the pressure effect of the CO_2 gets to be very near to the lowest possible, namely $P_{O_2} \times \frac{\alpha_{O_2}}{\alpha_{CO_2}}$. In a system like this one we must therefore strive to keep the gas volume less than 1% of the liquid volume. In most of our work it has been 0.5% or less.

With an increasing alkalinity the pressure effect of CO_2 will naturally get smaller and smaller. Exactly what it will be can be determined in the following way.

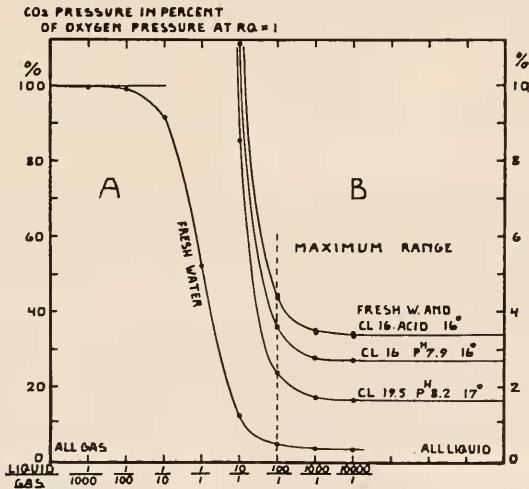


FIGURE 4. Pressure effect of carbon dioxide production in per cent of the pressure effect of an equal oxygen consumption when the gas-to-liquid ratio in the respirometer is varied. A. The entire curve for fresh water with ordinate units to the left. B. The lower part of three curves magnified ten times, with ordinate units to the right.

1. Determination of the CO_2 pressure

a. Principle. Increasing, known amounts of CO_2 gas are added anaerobically to a series of syringes containing 10 cc. sea water each. The CO_2 is dissolved quantitatively by chilling the syringes. The charged water is transferred with negligible gas loss to a long-necked 1 cc. flask in an accurately regulated water bath (Fig. 5). A micro reference diver is placed in the flask which is connected to a manometer and pressure regulating device (the same as for the respirometer chamber). The diver is brought to flotation and the pressure is read. This represents the total gas pressure plus the surface tension effect. A few cubic millimeters of strong NaOH are added from a microburette to make the sea water alkaline. The reference diver is again brought to flotation. The difference in pressure gives the CO_2 effect directly in mm. Hg.

b. Details of procedure. The flask is provided with a spiral depression so it can be vigorously stirred by twisting back and forth while still connected with the manometer. The diver is long so that the bubble will completely occupy the upper surface and hence will assume the same radius after collapse.

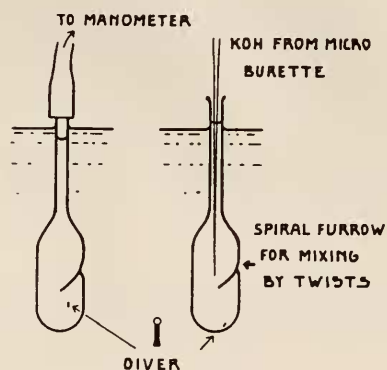


FIGURE 5. Reference diver technique for direct determination of CO_2 tension in liquid.

Flotation pressure is established in a few minutes and read. The chamber is twisted to effect stirring and flotation pressure is read again until it attains constancy. The burette tip is conveniently made from a pulled-out polyethylene micro tubing. It must be very fine (0.1–0.2 mm.). For 0.7 cc. sea water, 1.50 mm.³ 1 *N* NaOH sufficed.

The action of NaOH upon the surface tension of water has been found to amount to a change from 72.8 to 74.6 dynes/cm. at 20° at a concentration of 5% (Landolt-Börnstein, 1923, p. 240). The amount of NaOH added to the sea water gives approximately 0.01%, and hence the effect from change of surface tension is negligible.

c. Results. The pressure effect of CO_2 was determined for the different sea waters which were used (Fig. 6). Carbon dioxide has greater pressure effect in acid sea water than in fresh water, because the salinity lowers the CO_2 solubility. The pressure effect decreases rapidly with increasing pH. At pH 8.2–8.4 it is only half or less that of fresh water. From the curves the average retention of

the CO_2 can be calculated and hence the pressure effect of CO_2 for different gas-to-liquid ratios at different pH and salinity can be estimated (Fig. 4).

It will be seen that in all our experiments with sea water in the chamber, the pressure effect of the CO_2 has only amounted to 2–3% of the oxygen pressure effect. This correction is included in equation 8, but can usually be ignored because the chamber volume, and certainly the volume or weight of the respiring material, are known with less accuracy.

It is, however, important to know the magnitude of the CO_2 effect for the interpretation of any irregularities or bumps on the curves, and it is then well to realize that a maximum metabolic change in RQ from 0.7 to 1 will only show up as a 1% or less error in the oxygen consumption. Similarly, acid formation must be at least equivalent to the oxygen consumption on a molar basis before it begins to show up. In other words, the CO_2 binding properties of straight unbuffered sea water, such as used in our experiments, hold the CO_2 pressure effect

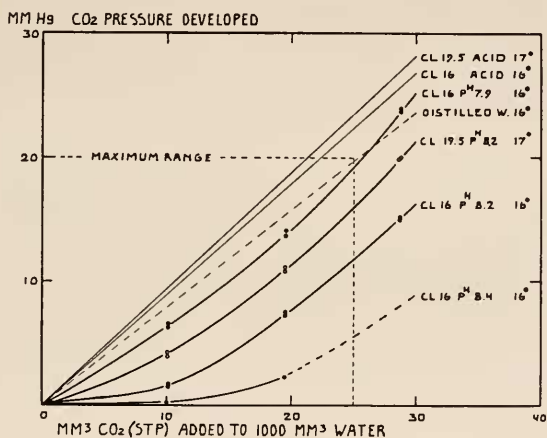


FIGURE 6. Pressure developed by adding known amounts of CO_2 to fresh water and sea water of varying pH and chlorinity.

down to a negligible amount. With less favorable gas-to-liquid ratio, and for special purposes, it may, however, be desirable to add a buffer to the sea water (Zeuthen, 1950).

The maximum amount of oxygen that can be used up in a chamber containing oxygen equilibrated water corresponds to some 2–3% of the water volume. The maximum CO_2 that can be metabolically produced at RQ 1 is then the same, namely around 20–30 volumes per 1000 water. The pressure effect of this can be read on Figure 6, and corresponds to 20 mm. Hg. It will usually be less than 10 mm. or corresponding to about 1% CO_2 at atmospheric pressure.

C. Diffusion characteristics of the system

This system, like all others, depends upon diffusion equilibrium between the gas and the water phase. The faster equilibrium is regained after a disturbance, the faster the system will be able to follow quick changes in respiration. Since the diffusion distances are very short in all directions (maximum 0.3 mm.) and

the volumes involved are very small, there is an exceptionally rapid tension equilibrium within the system.

This can easily be determined empirically. A blank is set up without respiring material and a 10–15 minute run is made in which the flotation pressure is accurately maintained and plotted every minute. A major disturbance is now introduced by putting 10 centimeters Hg pressure on the diver until within a minute the bubble has almost disappeared by dissolving into the water. The diver is again brought to flotation, and faster than it can be plotted, it regains its former flotation pressure within a half millimeter Hg. In other words, even major disturbances, involving the new generation of the gas bubble from the dissolved gas, are repaired within 30 seconds. Clearly, therefore, we can accurately record even very rapid respiratory changes with very little damping effect (Linderström-Lang, 1946).

D. Sensitivity

The sensitivity of the system depends upon several factors: (a) on the smallest increment or decrement of the bubble volume that will move the diver up or down, (b) on the magnitude of the pressure change necessary to produce this volume change, and (c) on the total pressure.

a. The method is essentially a "gravimetric" method, and therefore the greatest sensitivity will be obtained when the forces opposing the buoyancy changes are smallest. These involve the masses to be accelerated and the frictional forces. By using a reference diver instead of floating the whole respiratory chamber it is possible at once to reduce very drastically both the mass and the friction. The mass of the reference diver corresponds to around 150–200 $\mu\mu$. The micro Cartesian diver has a moving mass, when floating in the medium of specific gravity 1.3, of about 2.5 mg., *i.e.*, some 12,000 times more mass to accelerate than the reference diver. The viscosity of fresh (and sea) water for floating the reference diver is only half that of the flotation medium used for Cartesian divers.

b. The pressure necessary to convey a volume change to the diver gas depends upon the number of menisci to be moved or deformed. The reference diver has one meniscus, whereas the micro diver involves four. The present size reference diver can be held within about 0.2 millimeter Hg, and we have not so far tried a water manometer. During oxygen consumption, when the flotation pressure is steadily decreasing, the accuracy of defining the flotation pressure undoubtedly goes down, somewhat in proportion to the rate of pressure change.

c. The sensitivity bears a direct relation to the total pressure at which the diver is operated. This is apparent if we consider the least molar amount of gas added or withdrawn that we can measure. The resulting volume change will depend upon the ratio of the amount withdrawn to the total amount present in the system. The sensitivity related to molar amounts of gas, therefore, increases in an inverse proportion to the pressure. The higher sensitivity at lower pressures is clearly felt in the manipulation of the syringe.

E. Stability in time

The stability of the flotation pressure is measured by blank runs. It depends on several factors, the most important ones being: (1) constancy of the tem-

perature of the water bath and of the manometer, and (2) diffusion of gas into the chamber from the water trapped in the capillary space between the rod and the chamber. Other sources which, especially during respiratory runs, could cause a shift of the base line would be (3) changes in surface tension, (4) changes in water vapor tension and density of the medium, (5) changes in area of bubble attachment to the diver, with consequent change in diameter and pressure.

On Figure 7 are given curves of the stability of the flotation pressure as found in several series of blank runs in fresh water, sea water and some media. The stability of the flotation pressure seems largely independent of the chamber size.

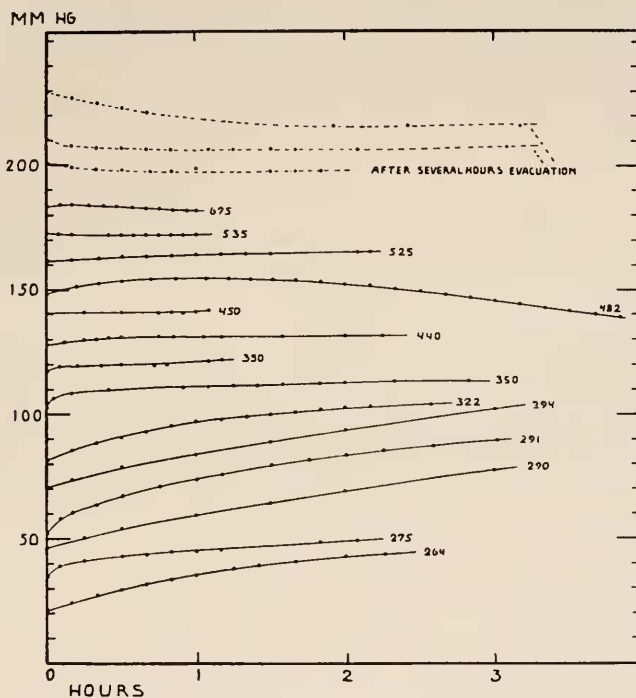


FIGURE 7. Stability of flotation pressure in blank runs. The number at the end of each curve plus 70 gives the flotation pressure in mm. Hg at the beginning of the blank run.

The stability in absolute volume units can therefore be taken as nearly proportional to the chamber size, *i.e.*, the factor F (p. 164). On the chart the factors actually went from 0.7 to 10, *i.e.*, one mm. Hg corresponded to from 0.7 to 10 $\mu\mu$.

1. Effect of temperature changes

From equation 1 we may calculate the temperature sensitivity of the system by knowing the amount by which the gas bubble, the solubility coefficient, the water vapor tension and the surface tension change with temperature. If we move from 20° to 21° C., α_{O_2} changes from 0.03102 to 0.03044, α_{CO_2} from 0.878 to 0.854, water vapor tension from 17.54 to 18.65 mm. Hg, and bubble pressure due to surface tension from 31.18 to 31.11 mm. Hg. If the bubble volume is

180 $\mu\mu\text{l}$ and the liquid volume is 20000 $\mu\mu\text{l}$ and the gas (O_2) is at 760 mm., this rise in temperature will change the pressure by 13.3 mm. Hg. A hundredth of a degree will change the pressure near to 0.13 mm. Hg.

It may similarly be calculated that when all the O_2 of the system (802 $\mu\mu\text{l}$) has been used up and 802 $\mu\mu\text{l}$ CO_2 have been produced, the resultant total pressure will be 20.8 mm. Hg at 20° . A one degree rise will then produce a 2.1 mm. increase in pressure, and 0.01° therefore 0.02 mm. increase.

If the system were all a moist gas phase it can similarly be shown that the same rise in temperature would raise the flotation pressure only 4 mm. We see thus that the temperature sensitivity increases: (1) the more liquid there is compared to gas, and (2) the higher the total gas tension of the system is.

The reason for the high sensitivity of a high liquid ratio lies in the fact that the all gas phase changes as $\frac{T}{T_1} = \frac{294}{293}$, whereas the all liquid phase tension

changes as the ratio of the solubility coefficients = $\frac{0.03102}{0.03044}$, which is a 5.6 times greater change. Similarly the pressure change of CO_2 in an all liquid system is 8.2 times as temperature sensitive as in an all gas phase, when the initial pressure is the same. In other words, the temperature effect on the solubility coefficients is much greater than on the gases themselves.

A compensating chamber is very commonly used in gasometric methods in order to cut down the temperature sensitivity of the system. It should be borne in mind, however, that a large stationary compensating chamber will not compensate for the temperature effect on the solubility coefficients of the gases. If the liquid-to-gas ratio in the respiration chamber is 10/1 or 2/1, a temperature change from 20° to 21° will leave the system uncompensated by, respectively, 40 and 10 mm. water. In such extremely sensitive systems as the Cartesian diver, it is therefore essential to keep an exceptionally accurate temperature control, especially so where the liquid-to-gas ratio is high.

We may conclude from the above calculations, which have been verified also by experiments, that we need to keep the water bath constant to within 0.01 degree to obtain stability of the flotation pressure within 0.2 mm. Hg. If we were to use a water manometer to be read with the same linear accuracy we would have to keep the temperature to within 0.001 degree.

It can easily be calculated that the mercury barometer must be kept within 2° C. in order to match the temperature sensitivity of the water bath, *i.e.*, $\pm 0.01^\circ$.

2. Diffusion of gases from the sleeve

The blank curves (Fig. 7) are arranged in a sequence, downwards according to the initial pressure reading, which is given in mm. Hg at the end of each curve. It will be seen that the lower curves, with a low flotation pressure, all show an initial rise, which in the first hour may amount to as much as 10 mm. Hg. This increase in flotation pressure diminishes, or disappears, as the flotation pressure approaches atmospheric pressure. The rise is due to gas diffusing in from the water trapped at one atmosphere's pressure in the capillary sleeve between the rod and the chamber wall. This can be proved by evacuating the loaded chamber for several hours, so that finally the sleeve water gets a low gas tension. When,

now, the flotation pressure is established, gas will diffuse back into the sleeve water from the chamber, producing a slight decrease in the flotation pressure (see the three upper curves).

We have not been able to demonstrate any measurable diffusion of gas *through* the sleeve. Even such drastic measures as changing the outside water from vacuum extracted to fully saturated water leave the flotation pressure undisturbed. Evidently diffusion through the capillary sleeve is exceedingly slow.

The pumping action produced by the slight volume changes in the bubble while adjusting the flotation pressure is much too small to effect any water renewal in the sleeve. It has so far been impossible even with gross changes in the bubble volume by vacuum to pump any dyed outside liquid into the chamber.

As the pressure during a run decreases the sleeve presumably continues to yield a small amount of gas proportional to the decrease in pressure. This will make a slight error in the chamber factor. Considering that the chamber factor and the cell volume are not very exactly known, the sleeve error is unimportant.

3. *Effect of changes in surface tension*

Any change of the surface tension of the diver bubble would change the bubble pressure, and this change would be reflected as such on the flotation pressure. During an experiment the medium is continuously changing with respect to gas tensions, pH, and organic and inorganic solutes due to the presence of the organism. Ferguson (1914) has shown that one atmosphere of CO₂ gas has very little effect on the surface tension of water, so that the maximum effect from CO₂ in our system would be entirely negligible. A negligible effect would also be produced by relevant changes in the concentrations of NaCl, KCl, urea, glycerine, sucrose, sodium phosphate, ammonium chloride (Landolt-Börnstein, 1923; McClendon, 1917). High molecular constituents of urine, bile and plasma may, however, significantly lower the surface tension. It would be of no significance for the method if a medium had a different surface tension from that of water, as long as it remained constant. It would not matter, either, if it showed a slow linear change, as this would only affect the factor converting pressure to absolute units of O₂ consumption. A sudden release of a surface acting substance (*e.g.*, protein) could give a sudden parallel displacement of the rate curve, but would then leave the rate unchanged. As surface acting substances are concentrated in the surface, they tend to exercise near their full action already at minute concentrations. If such substances were released periodically, it seems therefore likely that they would lead to only small periodic changes in the surface tension.

4. *Changes in water vapor tension and density of medium*

Only a change in the water vapor tension during the experiment will affect the results. A gradual change would be of no consequence, as both the absolute rate and the amount of respiring material would be less well known. Measurable sudden changes in water tension are even more unlikely than sudden changes in surface tension, and would tend to be counteracted by the density change.

5. *Changes in bubble diameter*

There is a possibility of a pressure change in the bubble if it changes its adhesion surface to the diver. This could happen if it does not fully occupy the whole cut surface.

The salt water diver has an oblique upper surface with no boundaries set for the bubble, which therefore could be assumed to vary in its attachment surface, and hence radius and pressure. In the experiments referred to on page 168, where the diver was subjected to gross transitory pressure changes, no changes in the final flotation pressures were detected, and hence there were no measurable changes in bubble radius. For tension determinations where it is necessary to generate the bubble twice, the diver is so long that the bubble will always completely occupy the cut surface.

III. PROCEDURE

1. *Preparation of sterile material and chamber*

It seems that bacterial contamination is a more conspicuous menace in this method than in macro methods. It is essential always to keep this in mind and try to avoid insidious errors from this source. Nutritive media especially, such as, for instance, hay tea or even sea water, must be very carefully sterilized and handled. Sea water was filtered through a porcelain candle (Selas, porosity 015) and then heated overnight in a thermostat of 70° C., allowed to stand at room temperature the next day, and then heated again to 70° C. The cool water was shaken with air and poured into two or three covered sterile Syracuse dishes.

All instruments and glass tubes, tips, vials, dishes and respiration chambers with rods must be boiled or autoclaved immediately before use.

The cell is transferred by means of a braking pipette into the water or medium of one dish, where it is allowed to sink or swim about. The pipette is rinsed several times in sterile water and the cell similarly transferred to two more dishes.

2. *Loading the chamber (Fig. 8)*

The medium is poured into a tube which is placed in a beaker of ice water and bubbled with moistened oxygen. The tube is kept loosely covered. After half an hour there will be enough oxygen in the water. The respiration chamber is "threaded" up on the suction tip, where it is held by the suction. The tube with bubbler is lifted up submerging the respiratory chamber, which thereby is filled and rinsed. The chamber is gripped by forceps while completely submerged, and the whole assembly is lowered as one piece down from the suction tip, leaving the respirometer chamber filled. The chamber is removed from the liquid and rolled up under a hook on the rubber stopper that serves as a chamber holder. It is imperative that there be no air bubbles sticking to the walls of the chamber. This is ascertained under the dissecting microscope. The cell is now transferred to a dish where it is allowed to sink through a centimeter or so of sterile medium and is then transferred into the opening of the chamber with a fine braking pipette. If the cell readily sinks, the chamber holder is put on its flat end. If the cell swims or does not freely sink to the closed end of the chamber, the chamber is placed in a simple cork centrifuge which is run very gently from a Variac. The chamber is then transferred back to the holder under the binocular.

At this point the diver is cut as described (p. 161), or picked up with a braking pipette from its storage drop.⁹ While still on the knife tip it is transferred to the

⁹ The diver filament will not stand heat sterilization. It may be wiped off with alcohol and water, or only water.

meniscus of the chamber where it will adhere to the surface. Using a fine needle, it is pushed into the liquid, unless deposited by a pipette. The stopper is held with chamber opening up and a slight tapping is usually enough to bring the diver down to the bottom next to the cell.

The rod is now rinsed off in the same medium from which the cell was taken. One of the drops adhering to it is touched to the chamber opening so the meniscus is convex and the solid rod is now inserted. In case the cell is a swimming organism like *Paramecium*, it may be quite a trick to get the rod in, using a combination of centrifugation and slight advances every time the animal moves in until the rod is finally seated. It is possible to some extent to feel how the rod should be rotated to seat deepest. Any quick pull back must be avoided,

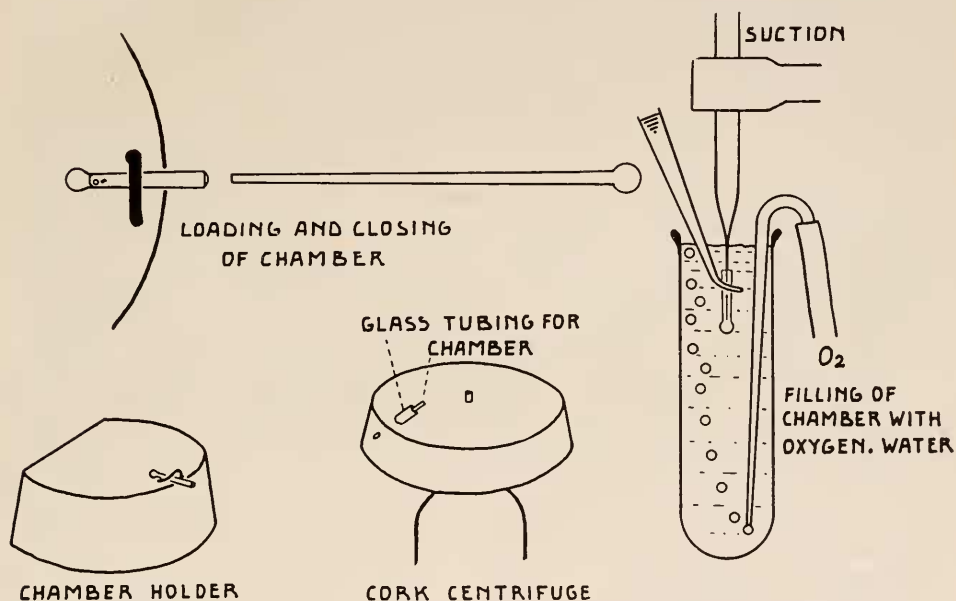


FIGURE 8. Operations involved in loading the chamber with oxygenated medium, cell and diver.

as this cavitates a bubble onto the diver and a positive flotation pressure may result.

The chamber, rod down, is now put into the chamber container, which is filled two thirds with the medium. The bubble fiber (p. 160) is put in and the container clamped in place on the water bath.

3. Measurement of chamber

At this time it is convenient to measure the chamber height and diameter by the ocular micrometer, and also the height of the diver from the base to the opening of the capillary hole. If a measurable cell is being used, like an egg cell, its diameter is also measured. It is often possible at once to calculate the chamber volume from the dimensions by using the chart mentioned on page 164, and from another chart (p. 175) read off the factor which translates mm. Hg to $\mu\mu l O_2$.

Especially if working with an assistant, one can then usually have the readings calculated and plotted during the run.

4. *Floating the reference diver (Fig. 3)*

For this step to succeed, it is necessary that the chamber and the rod be absolutely clean so the diver will not stick. With fresh water the walls are usually hydrophilic, a straight cut diver can be used and the flotation is easily accomplished. In sea water the walls become hydrophobic and flotation cannot be accomplished unless the bubble is kept from touching the glass walls. There are two precautions taken to make flotation possible: (1) shape of the diver; (2) orientation of the diver before generating the bubble.

The diver is shaped so that when it sinks and hits the bottom it tends to fall with the bubble surface upwards, and when it floats the bubble cannot touch the sides of the chamber.

To orient the diver the tube is rotated and tapped and tilted until the diver lands right side up on the bottom with the head end away from the wall and free from the cell. The tube is now turned so that the diver is seen in side view; it is connected to the manometer system and a slight vacuum admitted, enough to suck in the plunger of the syringe. The connection to the manometer is clamped. The vacuum is carefully increased by turning the syringe knob until the gas meniscus in the capillary of the diver moves out making a bubble. This will rapidly expand unless checked by the syringe. While lightly tapping the tube with a fine glass rod, the bubble is expanded until the diver rises and finally floats free from the bottom. It must not be allowed to hit the ceiling. When the pressure is stable, the clamp is barely opened and immediately closed, causing the diver to rise or sink, this being immediately counteracted by the syringe. If the diver sinks, the manometer pressure is lowered a few centimeters by the stopcock and the flotation pressure is again checked until the manometer pressure matches the flotation pressure and the clamp can be taken off. If the diver floats too near a wall, it can be brought near the middle of the chamber by tilting the chamber towards horizontal, bringing the diver up a little and then down when the chamber is again vertical.

If during these manipulations the diver gets stuck with the bubble against the bottom, top or side walls, pressure is put on to near atmospheric. The syringe plunger is pulled half way out and the clamp tightened. By applying overpressure with the syringe the bubble is made to disappear into the diver capillary and the diver is again free and can be re-oriented before a new flotation is attempted. If the diver itself sticks, it can usually be shaken loose if the chamber tube is removed from the water bath and given a violent shake down.

If the bubble inadvertently should dissolve completely, a new one usually cannot be formed by simple evacuation. There is still one chance left to save the run, namely, by cavitating a bubble onto the diver. To do this the chamber is removed from the tube and is held submerged in a dish of the medium. The rod is barely loosened very carefully and immediately pushed back in. This cavitates a bubble onto the diver which then often can be made to float. It is immaterial where the bubble forms on the diver. The flotation pressure is often high as a result of this procedure, sometimes even higher than atmospheric pressure. In such case air must be blown into the barometer flask and the

syringe must be manipulated with two hands, one continually pressing the barrel in, while the other regulates the motion.

5. *Recording of the flotation pressure*

With the reference diver afloat, the measurements can start, and readings can be taken at any suitable interval. The ocular scale is oriented in a vertical position, and the middle line of it is placed a little above the middle of the chamber. The distance between this line and the bottom of the chamber is maintained throughout the run, and the bubble is held so that the top of it touches the ocular line.

For most accurate readings, especially if close time intervals are desired, it is best to have an assistant start calling off every five seconds a half minute before reading time. During this period the diver is held at the mark very accurately and excursions of the pressure regulation are made as smooth and as small as possible. It is wise to check the water bath temperature at every reading and note it down.

6. *Re-setting of manometer pressure*

The syringe serves as fine regulation and its capacity is soon exhausted, especially when the total pressure is low. To re-set the pressure the stop-cock on the barometer flask is rapidly turned past the vacuum connection. This lowers the pressure by a few centimeters, and the diver rises. It is immediately brought back to the line by letting the plunger in. The procedure is repeated until the plunger is far enough in to last for some time.

7. *Removing cell and diver from the chamber*

It is sometimes useful to be able to remove the cell and diver from the chamber, so that the same run can be repeated later or with a different medium, or it may be desirable to measure the cell volume or other properties of the cell that was used. In order to do this, the chamber is taken out of the tube and is held in a Petri dish containing the medium. With the sleeve covered by the liquid the rod is very gently pulled out, filling the chamber with the medium. The chamber is then placed, opening down, in a small dish of the medium, letting the diver and the cell fall out.

8. *Calculation of results*

It is customary in micro respirometry to recalculate the readings as increasing from the first one, which is called zero, by subtracting all the subsequent readings from the first. In a detailed study all these figures, showing the change in pressure as a function of time, must be recalculated to give $\mu\mu\text{l O}_2$ consumed. This is done by first calculating the liquid volume as described on page 164 and then using the formula (8). If the diameter of the diver filament is kept constant and the diver length reasonably constant, a great deal of calculation can be saved by computing the factor for four situations, using two different chamber volumes, of, say, 20,000 $\mu\mu\text{l}$ and 100,000 $\mu\mu\text{l}$, and two different diver lengths, one with height of the diver equal to the diameter and the other equal to one and a half times the diameter. A graph is prepared with the factors on the ordinate and V_L on the abscissa. The four points are plotted and the pairs connected with

parallel lines. In the relevant region the function (equation 8) is very nearly a straight line, and the factor is found by interpolation.

This chart can of course only be used in series where the temperature, salinity and diver diameter are kept the same.

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SUMMARY

1. A method for micro respiratory studies is described with a sensitivity down to 0.2 millionth of a cubic millimeter and a stability of 10-0 millionths of a cubic millimeter an hour. It is basically a constant volume method, where a minute reference diver is introduced into the chamber together with the cell. A bubble is generated on the diver until it just floats. The change in flotation pressure multiplied by a factor of known constants gives the oxygen consumption. The CO₂ is effectively held in solution by the high liquid-to-gas ratio. The system has been used to study the respiration of single protozoans and to follow in detail the oxygen consumption during cleavage of single eggs of several species, which will be reported elsewhere.

2. A new micro method is described for direct determinations of gas tensions in liquids using the reference diver.

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